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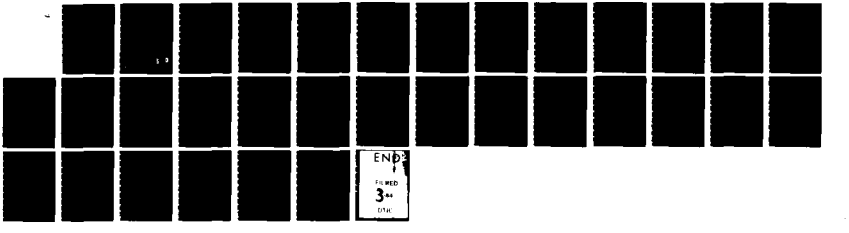

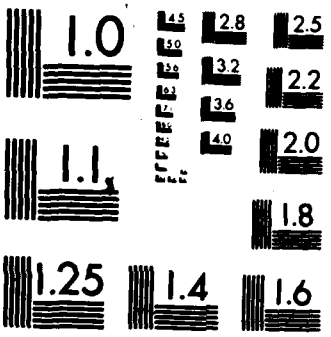

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The degree of nutrient limitation and indeed the limiting nutrient involved may modify the response of the community to toluene. A nutrient-limited community may be more affected by toluene than a non-nutrient-limited one. In a nitrogen-limited community, algal growth may be stimulated.

Differential toxicity may affect the outcome of species competition for nutrients. The species that is least susceptible to toluene may obtain a numerical advantage, at least temporarily, while the other species are inhibited. The duration of this advantage may depend on the rate to which nutrients are returned into the medium.

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CAN THE SHORT-TERM TOXICITY OF WATER-SOLUBLE JET FUEL HYDROCARBONS PRODUCE LONG-LASTING EFFECTS IN LAKE PLANKTON COMMUNITIES?

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INTRODUCTION

The low molecular weight aromatic hydrocarbons such as benzene, toluene, and xylene have often been suggested to be the primary toxic components of spilled oils and fuels. However, because of their low solubility in water and their high volatility, they are thought to remain in the water only a matter of hours or days. Their toxicity should be limited to only a brief time after the initial spill. Indeed, Coffey et al. (1977) found that allowing an oil-water extract of No. 2 fuel oil to stand for 72 hours prior to the introduction of the algae significantly decreased its toxicity. In refined fuels such as gasoline, kerosene, or jet fuels, where these aromatics are the dominant water-soluble components of the fuels, it might be concluded that spills of these fuels would have only a temporary effect on an aquatic environment.

Algal bioassays appear to bear out these assumptions. Numerous studies done with the pure hydrocarbons or with oil-water extracts document that the major impact of these compounds is an increase in the lag phase of the growth curve. The duration of this inhibition is dependent on the time taken for the compound to disappear from the water (Soto et al., 1975), and on the initial concentration of the aromatic compound (Kauss and Hutchinson, 1975). Once the compound declines to a non-toxic level, growth of the algae resumes at rates equal to the non-treated controls. At high concentrations, the aromatic compound may inhibit photosynthesis, but at low concentrations photosynthesis may be stimulated (Prouse et al, 1975; Dunstan et al, 1975; Parsons and Waters, 1976; Karydis and Fogg, 1980). Final biomass apparently is not affected (Karydis and Fogg, 1980). Different species of algae may, however, exhibit varying degrees of susceptibilty to these hydrocarbon compounds (Dennington et al., 1975; Coffey et al., 1977)

The intent of the present study is to determine to what extent the results of the bioassays do not reflect the possible effects of the spilling of a refined fuel in a natural ecosystem. Implicit in this work is the concept that the natural aquatic ecosystem has a degree of biotic structure, which, once disturbed, does not return readily to its prior conformation. If, for example, the algal species composition is primarily determined by the species and density of herbivores, then a change in the herbivore community might result in a different algal community, and the rate of recovery of the algal community would be dependent on the recovery rate of the herbivores, not on the rate of loss of the hydrocarbon from the water.

The two aspects studied in the present study were: (a) to what extent the ambient nutrient regime affects the recovery of algae, and (b) to what extent does the presence of more than one species of algae influence the recovery of algae. Lakes and ponds are thought to be nutrient limited,

and nutrient stress may alter the response of the algae to toluene additions. Secondly, as algae occur in multi-species associations, toluene may not affect each individual species of algae separately, but may produce unpredictable effects related to the interactions between the algae themselves.

METHODS

The algae used in the experiments were Selenastrum capricornutum or Microcystis aeriginosa obtained from the Richard Starr Algal collection or Ankistrodesmus sp. or Chlorella sp. from the Carolina Biological collection. The cultures were unialgal but not necessarily axenic, although precautions were taken to minimize bacterial contamination. The algae were cultured in either Bold's Basal Medium (Bischoff and Bold, 1963) or in the U.S.E.P.A. Algal Asssay Medium (Miller et al., 1978). Bold's Basal Medium (BBM) is a relatively rich medium used for the maintenance of cultures. In this medium, the algae eventually become nitrogen limited, as the N:P ratio of the medium is only 0.76. The Algal Assay Meduim (AAM) is far more dilute, and is phosphorus limiting, having a N:P ratio of 23:1.

The bioassay experiments were conducted in acid rinsed, autoclaved, 250 ml erlenmeyer flasks containing 50 ml of medium. In each test approximately 2,000 to 3,000 cells taken from a 7 day old culture were inoculated into each flask. Experiments were performed at room temperature (24° C $\pm$ 2°) under continuous "cool white" fluorescent lighting. Each flask was stoppered with a cotton plug wrapped in gauze, which allowed gas exchange with the atmosphere. The flasks were placed into a closed, transparent Lucite box attached to a vacuum system to remove any toluene from the laboratory.

To produce nitrogen and phosphorus limited cultures, cells of Selenastrum capricornutum were grown in AAM media until the stationary phase of growth was reached. These phosphorus-limited cells were then used to inoculate cultures in similar media having N:P ratios of approximately 23:1 or 1:1. The 1:1 ratio was achieved by decreasing the concentration of $NaNO_3$ in the AAM medium. These algal stocks were again allowed to grow to stationary phase. New cultures of the same N:P ratios were then grown using inoculum from these stock cultures. Cells from these cultures were used in the subsequent experiments.

Toluene was used as the model aromatic hydrocarbon. It was chosen because it is often the chief water-soluble component of jet fuels and other refined fuels. Because it is in the liquid phase and has moderate volatility, it is easier to use than napthalene which often must be used together with a solvent (Vandermeulen and Ahern, 1976). The toluene used was 99 mol % pure (Fisher Chemical).

If the algae were already growing in the culture, the toluene was added by dropping a known amount into the medium and gently swirling the flask to mix it into the medium. The amount added was sufficient to assure near saturation of the medium. In cases where the toluene was added to the medium prior to the addition of algal cells, the following

technique was used. Ten ml of toluene was added to the algal medium in a separatory funnel. The mixture was shaken, allowed to stand for at least 24 hours, and the toluene-saturated medium drawn from the bottom of the funnel.

Algal density was enumerated by either ot two methods. In some experiments the optical density of the culture was measured at 678 nm using a Bausch and Lomb spectrophotometer, Model 88. This method was discontinued when it was thought that the turbidity was kept high after toluene additions because of the interference of dead or inactive cells. In subsequent experients direct cell counts were made using a Neubauer hemacytometer. As will be mentioned later, this method also could not discriminate between dead and living cells. In vivo fluorescence was tried, but the fluorescence of the toluene interfered. We also tried using a vital stain (methylene blue), but the discrimination between dead and living cells was still not sufficient. Chlorophyll measurements were considered, but the method would take too much of the culture, and the loss of chlorophyll would not necessarily mean that the cells had been killed. It may be that fluorescence microscopy or ATP measurements may be needed in order to obtain an accurate measure of algal mortality.

RESULTS

Nutrient Inactivation

In a first experiment to demonstrate differential susceptability to toluene, three species of algae, Selenastrum, Ankistrodesmus, and Chlorella were grown in BBM medium. After five days of growth, half of the flasks (3) for each species were spiked with 0.1 ml of toluene. The flasks were shaken for 10 seconds to disperse the toluene in the medium. Optical density of the cultures was measured with a spectrophotometer.

The results (Figures 1,2, and 3) suggested that all three species experienced a slowing of growth and some decrease in numbers after toluene addition. Selenastrum quickly recovered and grew at rates equal the toluene-free cells, finally reaching the same final biomass. Ankistrodesmus and Chlorella however, neither grew at the same rates nor did they achieve the same final biomasss as the control cultures.

Although this experiment might be evidence for differences in susceptibility to toluene, the results could also be explained as differences in the rates of uptake of nutrients from the medium within the flasks. If Selenastrum had a lower nutrient uptake rate than did Ankistrodesmus or Chlorella, then there would still be considerable amounts of nutrients remaining in the medium for the growth of the surviving cells. To test this possibility, phosphorus and nitrogen were added to separate flasks of the toluene-treated Chlorella cultures. Nitrogen additions produced no observable effect, but there was an immediate increase in the growth in the cultures to which phosphorus was added (Fig. 3). The results suggested that phosphorus had been taken up rapidly by Chlorella, and perhaps Ankistrodesmus, in the early stages of growth, and after toluene addition killed or inactivated the cells, there was not sufficient phosphorus remaining in the medium to allow the complete recovery seen in Selenastrum.

To test the possibility that nutrients taken up by the algae are not made available after toluene addition, cells that were already nutrient limited were spiked with toluene. If nutrients were released from these dead or inactive cells, then the cultures would recover. If no nutrients are made available, the cultures should not change.

Each of 12, 250 ml sterile, acid-rinsed erlenmeyer flasks containing one hundred ml of AAM medium with an N:P ratio of 1:1 were aseptically inoculated with 4.5×10^6 cells of N-limited Selenastrum. This resulted in initial concentration of 4.5×10^3 cells in each flask. Cotton plugs were used to close the flasks yet allow gas exchange. Each flask was gently swirled daily. Cell counts were made with a hemacytometer until stationary phase was reached (16 days). At stationary phase each flask was spiked with 0.15 ml of toluene. The flasks were

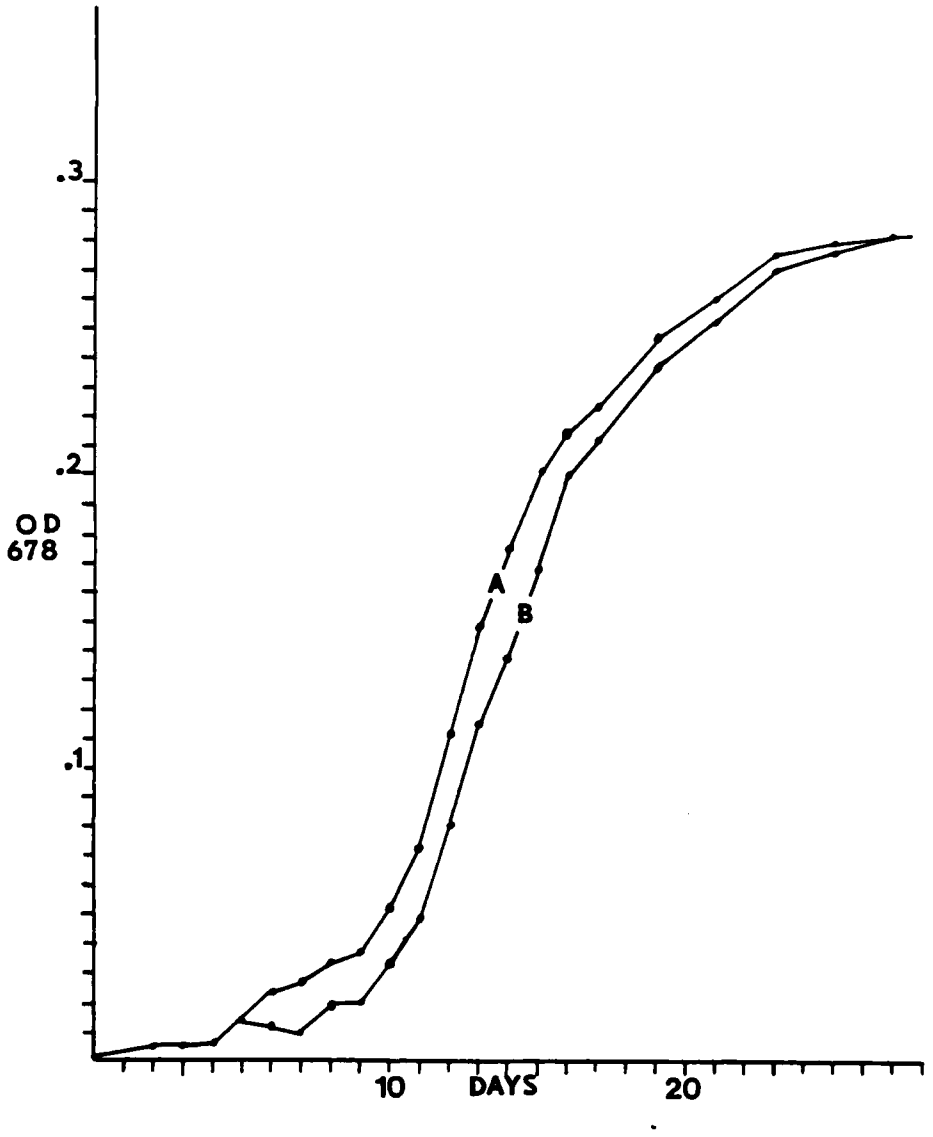

Figure 1. The effect of a saturating concentration of toluene on the growth of Selenastrum. Curve A is the control; curve B is exposed to toluene.

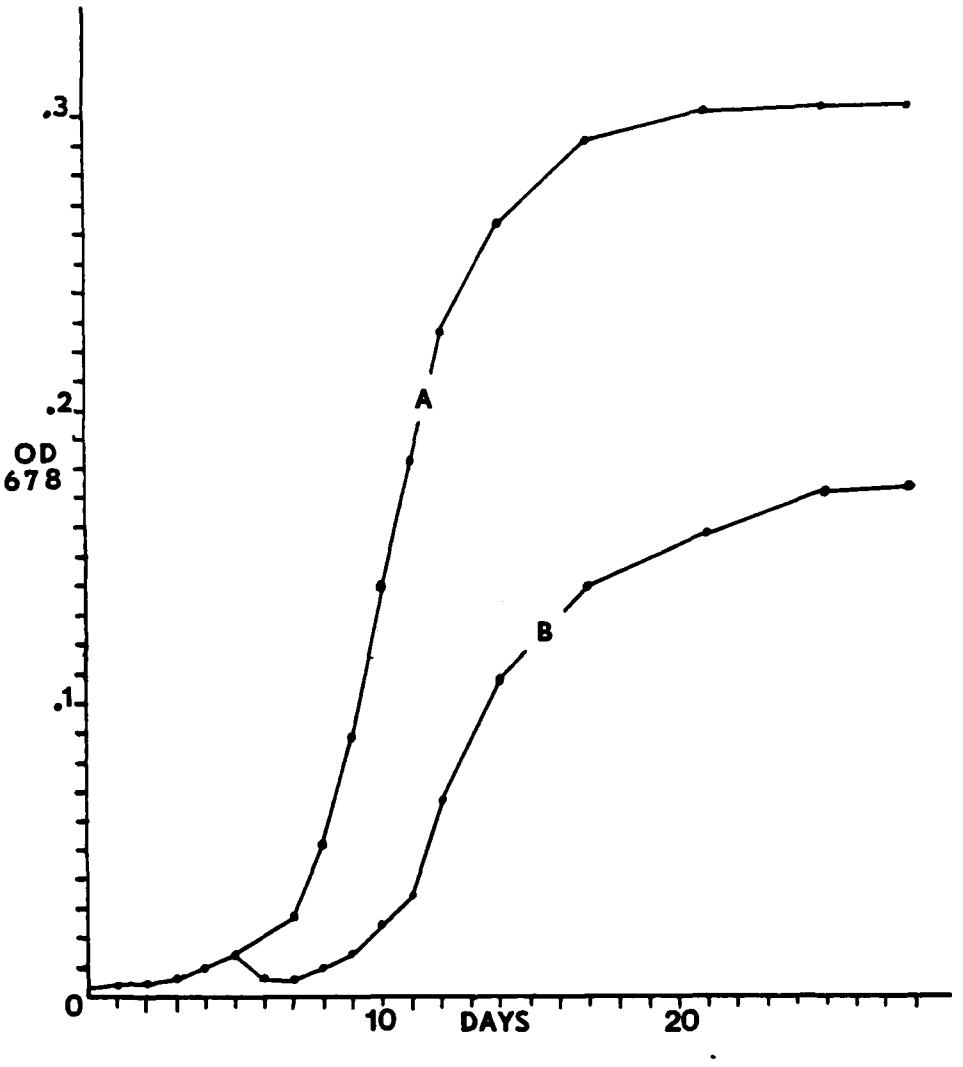

Figure 2. The effect of a saturating concentration of toluene on the growth of Ankistrodesmus. Curve A is the control; curve B is exposed to toluene.

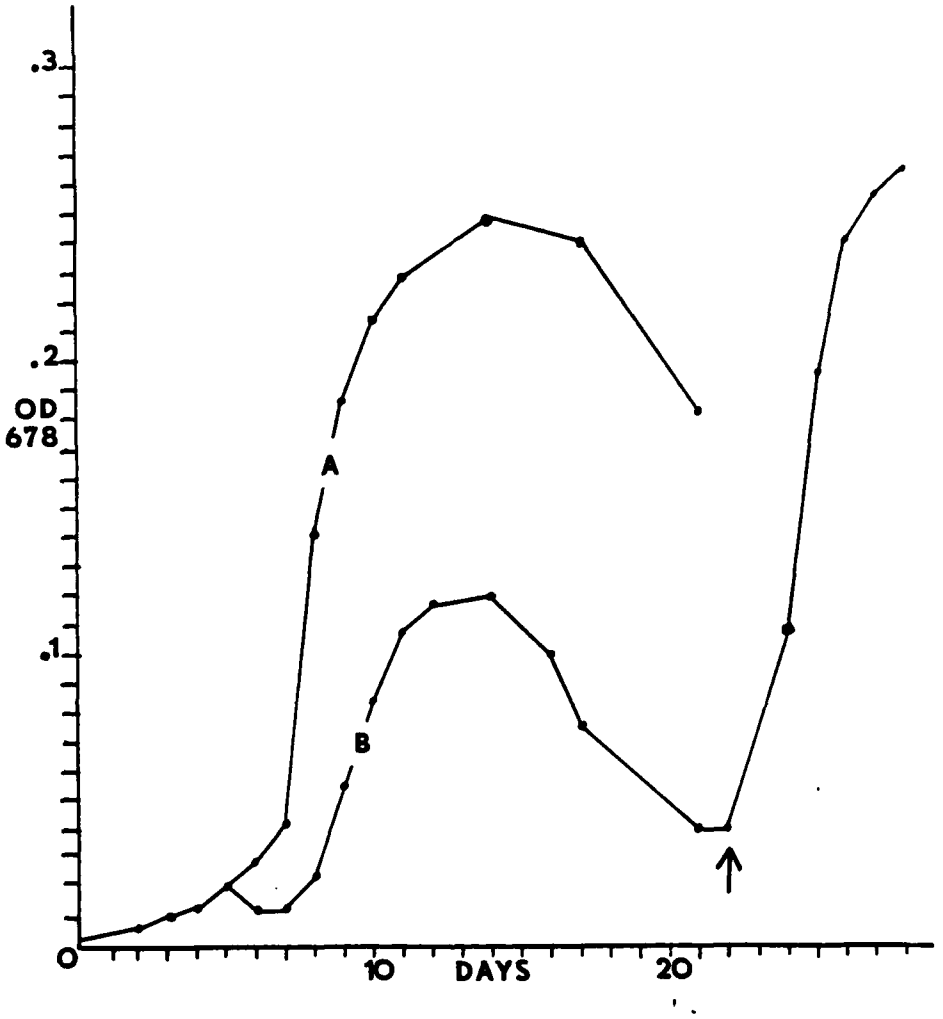

Figure 3. The effect of a saturating concentration of toluene on the growth of Chlorella. Curve A is the control; curve B is exposed to toluene.

swirled for 1 minute to assure mixing of the medium with toluene. The concentration of toluene in the medium was near 100% saturation. Since cotton stoppers were used, most of the toluene was expected to evaporate within a 24-48 hour period, but no measurements of toluene concentration were made.

Daily cell counts were made after the treatment. Four days after the addition of toluene, nutrients were supplied to the flasks by the addition of either 0.15 ml of $NaNO_3$ or 0.15 ml of K_2HPO_4. Four flasks served as controls (no additional nutrients were added). The $NaNO_3$ and K_2HPO_4 stocks were made according to EPA-AAM medium specifications and the amounts added to the flasks supplied a surplus of the respective nutrients to the flasks.

The green color of the algae in the flasks disappeared almost immediately (several seconds) following the addition of toluene. However, subsequent counts indicated only a small decrease in cell numbers (Figure 4). This suggested either live cells could not be discerned from dead cells or chlorophyll was broken down but cells did not die (or did not lyse). Because of this, cell counts were discontinued. After 20 days, 2 flasks of each treatment group were spiked with 3.6×10^5 cells to determine whether the medium was still toxic.

The observations summarized in Table 1 show that only flasks supplied with excess nitrogen returned to a green color. No regrowth was seen in those flasks to which no additional nutrients had been added or to which phosphorus had been added. The first flask to turn green had not been spiked with excess cells while the 2 flasks spiked with extra cells returned to a green color 15 days after the cells had been added. The growth in these flasks was a result of increased cell numbers in these flasks, and not just the return of chlorophyll pigments to already existing cells. Cell numbers in these flasks were greater than those in colorless flasks although both were lower than counts made at the original stationary phase and immediately following toluene treatment (Figure 4).

The results of these experiments suggest that, at least under these culture conditions, nutrients taken up by the growing algal cells are not released after a toluene injection. If a culture or natural environment is nutrient limited, recovery is dependent on the re-supply of that limiting nutrient to the medium, not to the initial toxicity of the toluene. Interestingly, although cells grown in BBM will ultimately be nitrogen limited because of the very low N:P ratio of the medium, species of algae that apparently have very fast phosphorus uptake rates appear to be modifying the N:P ratio towards P-limitation in the early days of population development. When toluene is added to these algae, phosphorus rather than nitrogen becomes limiting with the death or inactivation of the cells.

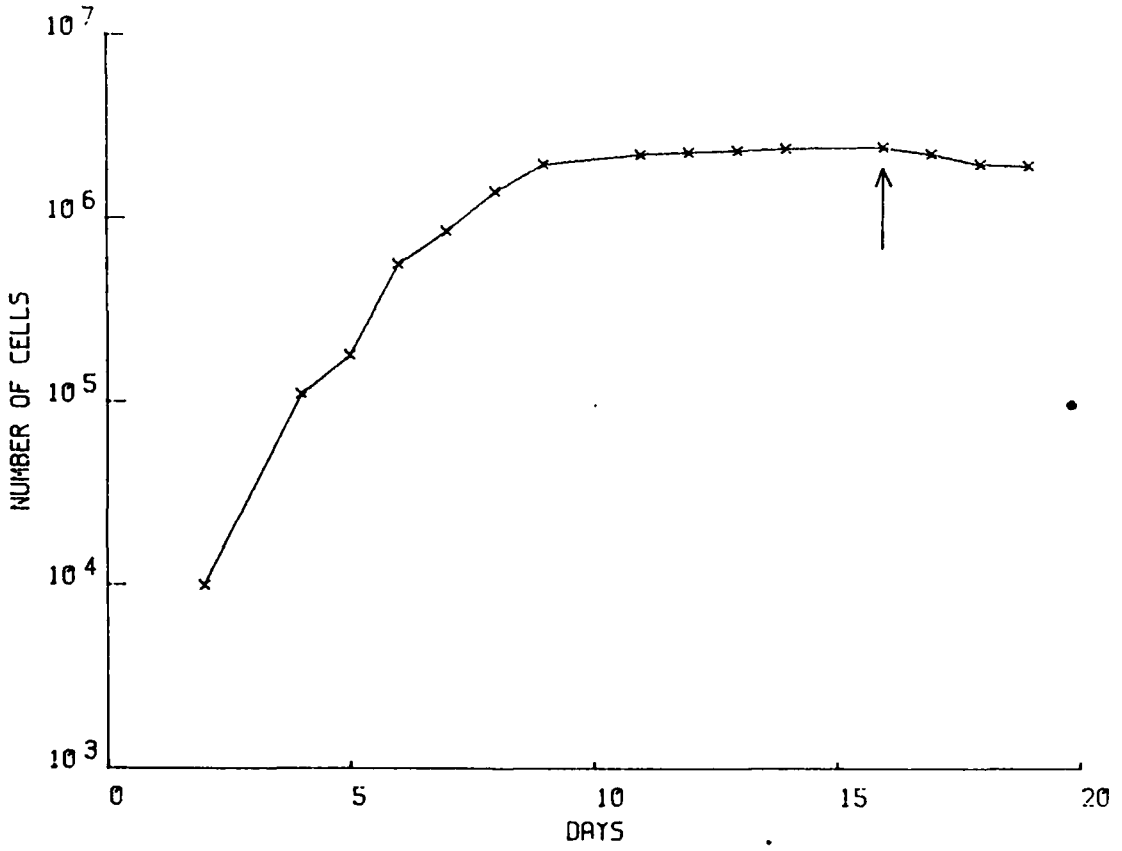

Figure 4. The effect of a saturating concentration of toluene on the numbers of cells (number per ml) of Selenastrum. The arrow indicates when the toluene was added.

Table 1. The effect of toluene addition and subsequent nutrient addition on the recovery of nitrogen-limited cultures of Selenastrum capricornutum . All flasks had been spiked with 0.15 ml of toluene 4 days prior to addition of nutrients.

Flask	Nutrient Added	Spiked with 3.6 x.105 cells	Length of time from addition of nutrients until green color noticed in flasks
1	N	-	51 days
2	N	-	23 days
3	N	yes	35 days
4	N	yes	35 days
5	P	-	-
6	P	-	-
7	P	yes	-
8	P	yes	-
9	-	-	-
10	-	-	-
11	-	yes	-
12	-	yes	-

Toluene and Interactions Among Algae

It is generally thought that algae in lakes and ponds are competing to some extent for nutrients. There are a number of nutrient-based models to explain the seasonal succession of algae and the co-existence of the many algal species often found at one time. If species are differentially susceptible to toluene, then one might expect that, at least temporarily, competitive advantages might shift and other species might predominate. This effect would not be simply differential survival, but the surviving species gains a competitive nutrient advantage through numerical superiority.

In our first experiments described above, it appeared as if those species that were most susceptible to toluene also were those that may have had the fastest nutrient uptake rates. A test of their susceptibility to toluene was confounded by the amount of nutrients remaining in the medium. To test susceptibility without the nutrient effect, a standard algal bioassay was performed where the toluene was added to the medium prior to the introduction of the algae.

Standard AAM medium was placed in a 1000 ml separatory funnel and 10 ml of toluene added. The mixture was shaken, and allowed to stand for 24 hours before use. This toluene saturated AAM medium was then diluted with standard AAM medium to produce toluene concentrations of 100%, 50%, 25%, and 12.5% of saturation. Flasks containing 100 ml of these media were inoculated with approximately 6000 cells per ml of either Selenastrum or Microcystis. Cells were counted with a hemacytometer. Three replicates of each toluene dose were made.

Growth of Selenastrum (Fig. 5a) was inhibited for 10 days at 100% saturation and for 2 days at 50%. After the initial inhibition, the growth rates approximated that of the control. Lower concentrations of toluene had no apparent effect on growth rate or final biomass. Growth of Microcystis was inhibited for approximately 11 days at toluene concentrations of 100% and 50% saturation (Fig. 5b). Once growth resumed, growth rates were equivalent to the controls and the same final biomass was reached. Microcystis appeared to be almost twice as sensitive to toluene than was Selenastrum .

The two species were then grown together to examine if differential toluene toxicity affected interactions between algal species. The results (Figure 6) indicate that, with no toluene present, there was no effect of the growth of one species on the biomass of the other, as the final numbers for each species were similar to the numbers achieved in the tests with the species alone. Toluene concentrations of 12.5%, 25%, and 50% saturation caused little deviation from the single species tests, although at 12.5% and 50%, Selenastrum had higher final numbers than found in the tests with that species alone (Figs. 7,8, and 9). The growth rates of Selenastrum appeared to be slightly decreased when grown together with Microcystis, while Microcystis growth rates appeared to be slightly stimulated . At toluene concentrations of 100% saturation there was a

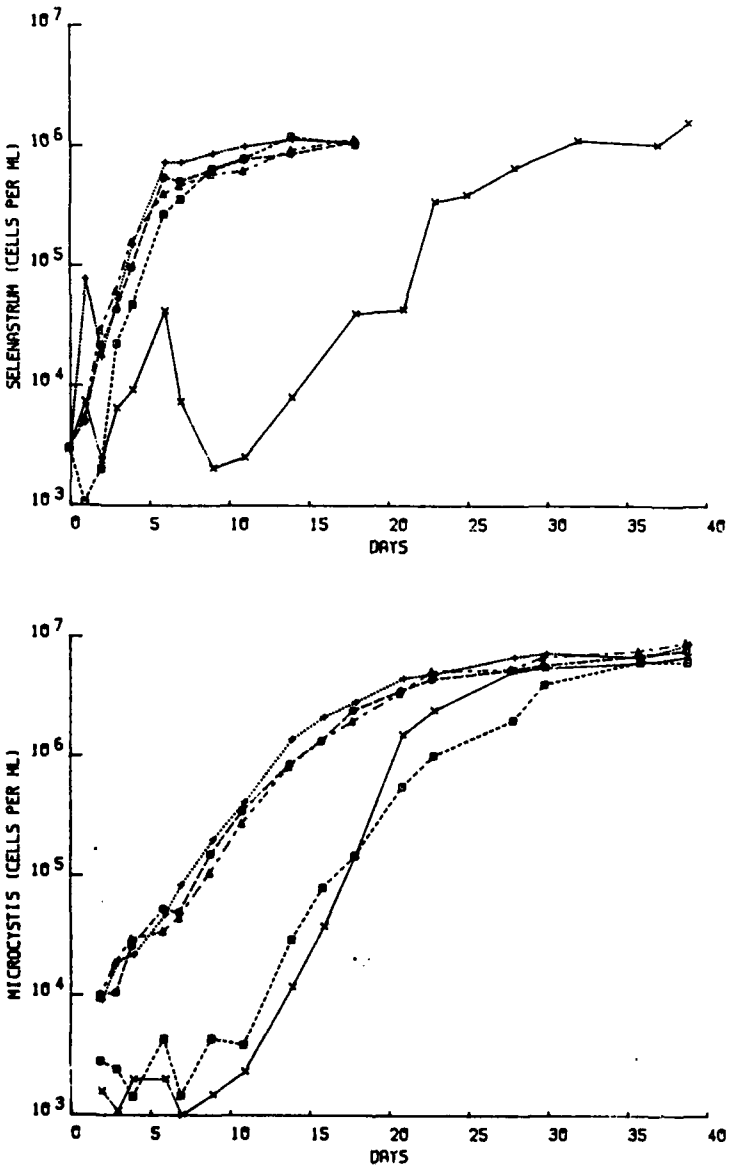

Figure 5. The growth of Selenastrum (upper graph) and Microcystis (lower graph) at various initial concentrations of toluene. The symbols represent the following concentrations: "+", control; triangle, 12.5%; circle, 25%; square, 50%; "x", 100% of saturation.

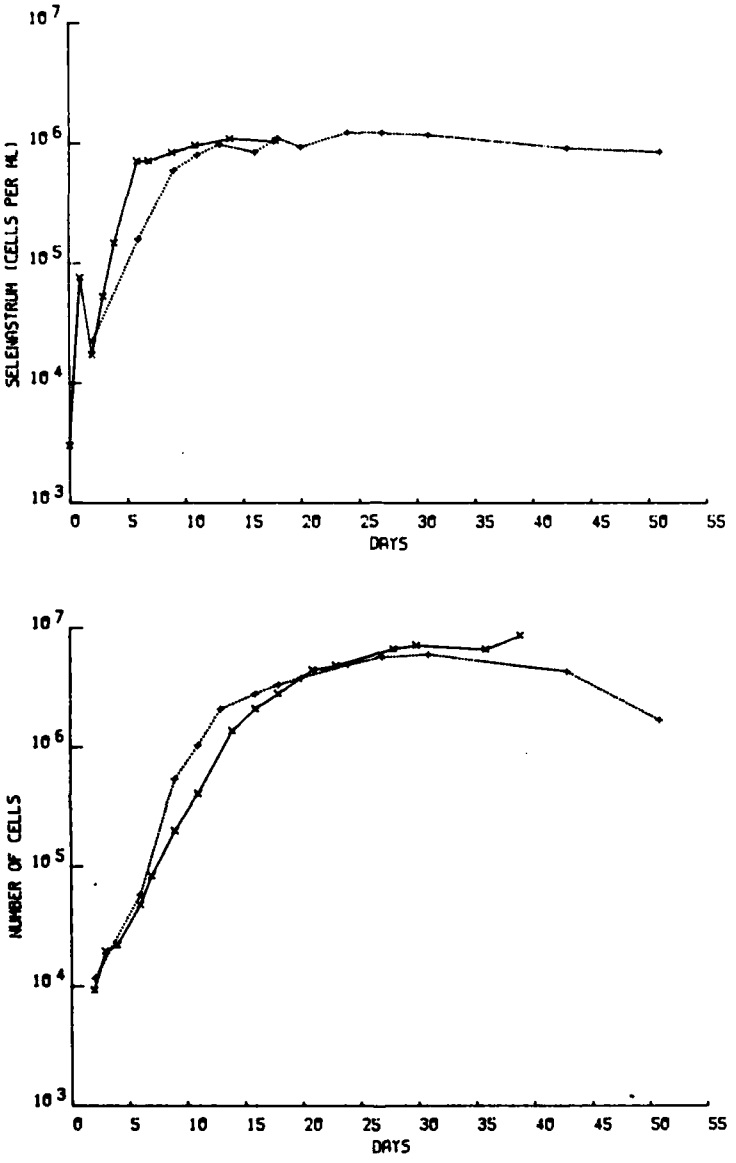

Figure 6. The growth of Selenastrum (upper graph) and Microcystis (lower graph) alone (+) and together with the other species (x). No toluene was present.

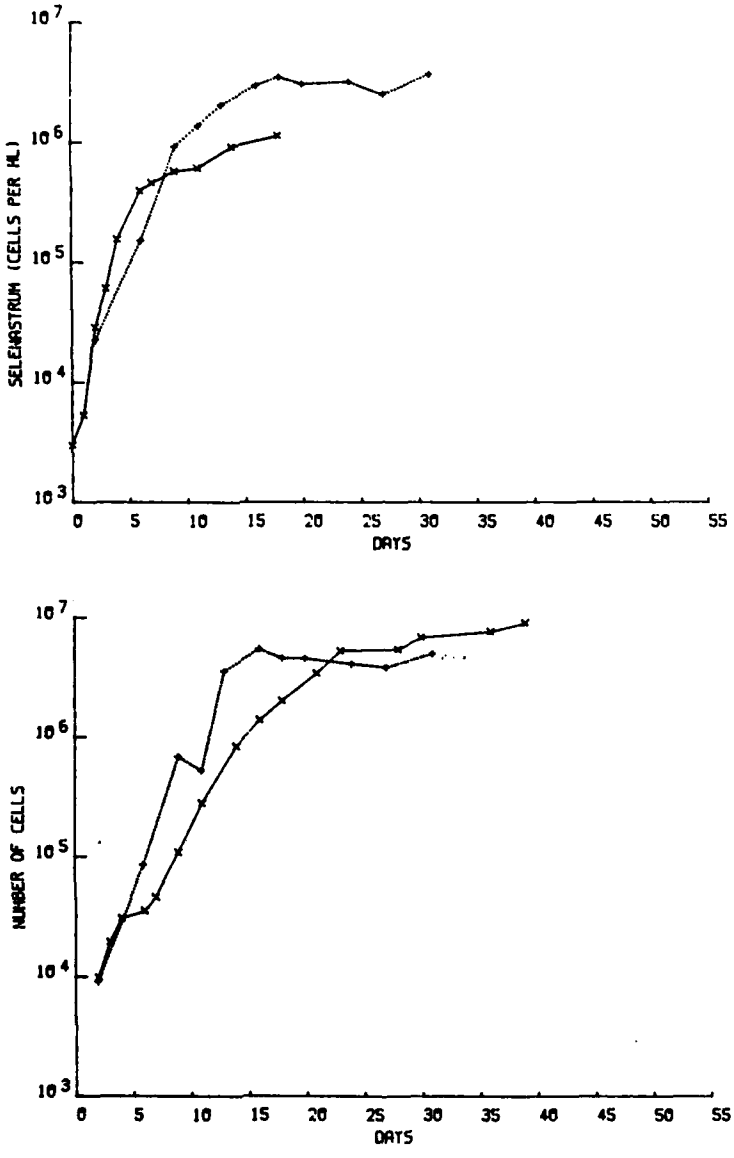

Figure 7. The growth of Selenastrum (upper graph) and Microcystis (lower graph) alone (+) and together with the other species (x) in a toluene concentration of 12.5% of saturation.

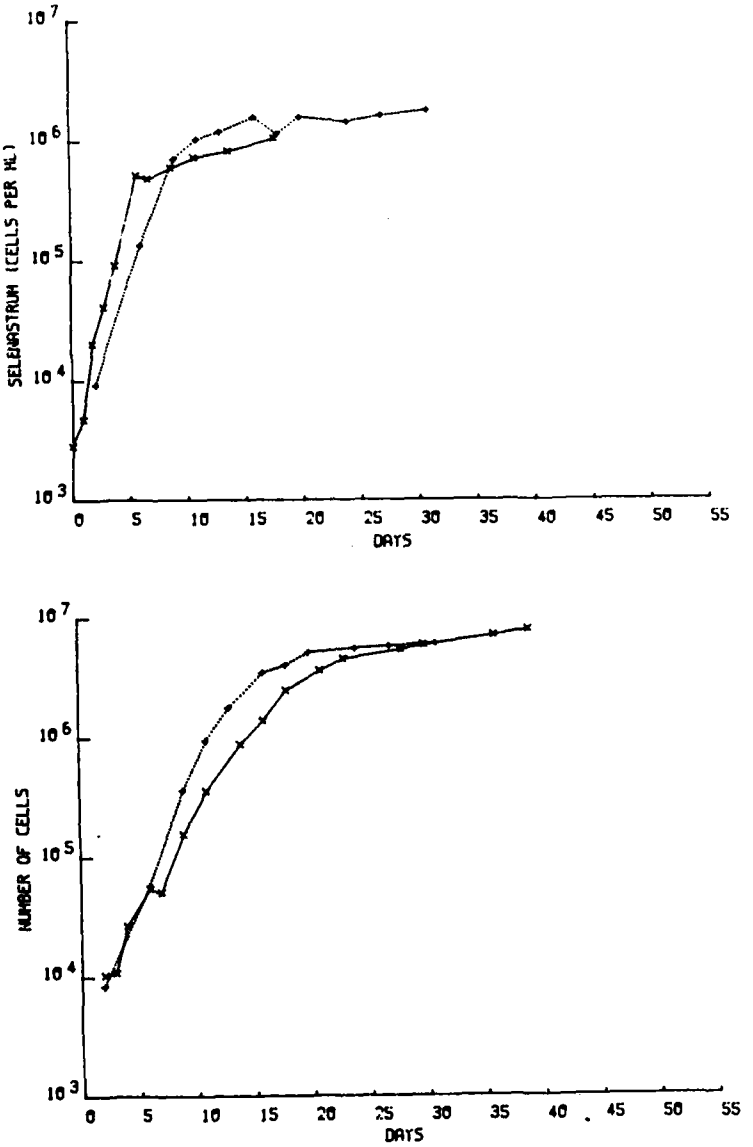

Figure 8. The growth of Selenastrum (upper graph) and Microcystis (lower graph) alone (+) and together with the other species (x) in a toluene concentration of 25% of saturation.

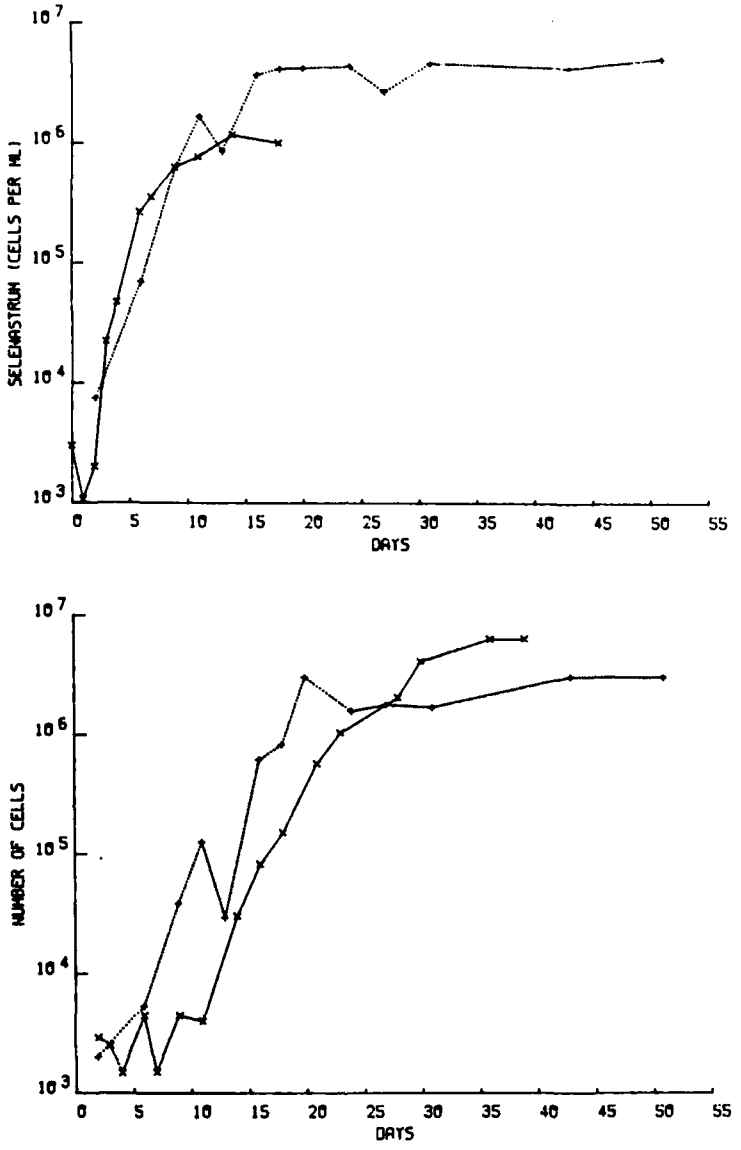

Figure 9. The growth of Selenastrum (upper graph) and Microcystis (lower graph) alone (+) and together with the other species (x) in a toluene concentration of 50% of saturation.

marked difference in the relative growth of the two species (Fig. 10). As in the single species experiments, Selenastrum recoverd more rapidly than did Microcystis and began to rapidly grow while Microcystis was still inhibited. Perhaps the uptake of nutrients by the growing Selenastrum caused the decreased growth rate of Microcystis.

Effects of Toluene on Nutrient Limited Cells

If nutrient availability stresses the algal cell, then one might hypothesize that the degree of toluene toxicity may be dependent on the degree of nutrient limitation of the algae. To test this possibility, saturating concentrations of toluene were added to algal cultures at various stages of their growth curves and the resulting growth patterns observed.

In the first experiment, Selenastrum was grown in Bolds Basal Medium (BBM). Pairs of cultures were spiked with 0.1 ml of toluene on days 1,2,3,4,5,6,11, and 16. Optical density was measured on a spectrophotometer.

Exposure to toluene produced different decreases in optical density and in the duration of the time for recovery dependent on the age of the culture (Fig. 11). The older the culture, the greater the proportional decrease in optical density and the longer the lag period.

The experiment was than repeated using the Algal Assay Medium (AAM). In this case a greater portion of the growth curve was used for the toluene additions. The results (Fig. 12) again indicate a proportionately greater decrease and larger lag period as the age of the culture increases. Cultures near stationary phase did not recover. It was possible, as with the earlier experiment with Chlorella, that the growth response was simply a function of nutrient availability rather than of differing susceptibility to toluene. To test this possibility, phosphorus was added to one flask of each treatment pair three to four days after the toluene addition. No change in algal density or recovery rate was noted in these flasks. As these cultures should have been phosphorus limited, especially at stationary phase, the lack of response to phosphorus indicates that nutrient unavailability was probably not involved. It is possible, however, that a majority of the cells were killed by the toluene, and the duration of the "lag phase" in actuality reflects the time necessary for the cells to regrow to their former density.

Another approach to determining the effect of nutrient limitation on toluene toxicity was to use nitrogen or phosphorus limited cells, and to grow them in several concentrations of toluene. The experiment would detect any differences in the response of nitrogen or phosphorus limited cells to toluene addition.

Sterile 125 ml flasks containing 50 ml of a mixture of AAM media (N or P free) and toluene were used. Concentrations of toluene in the flasks

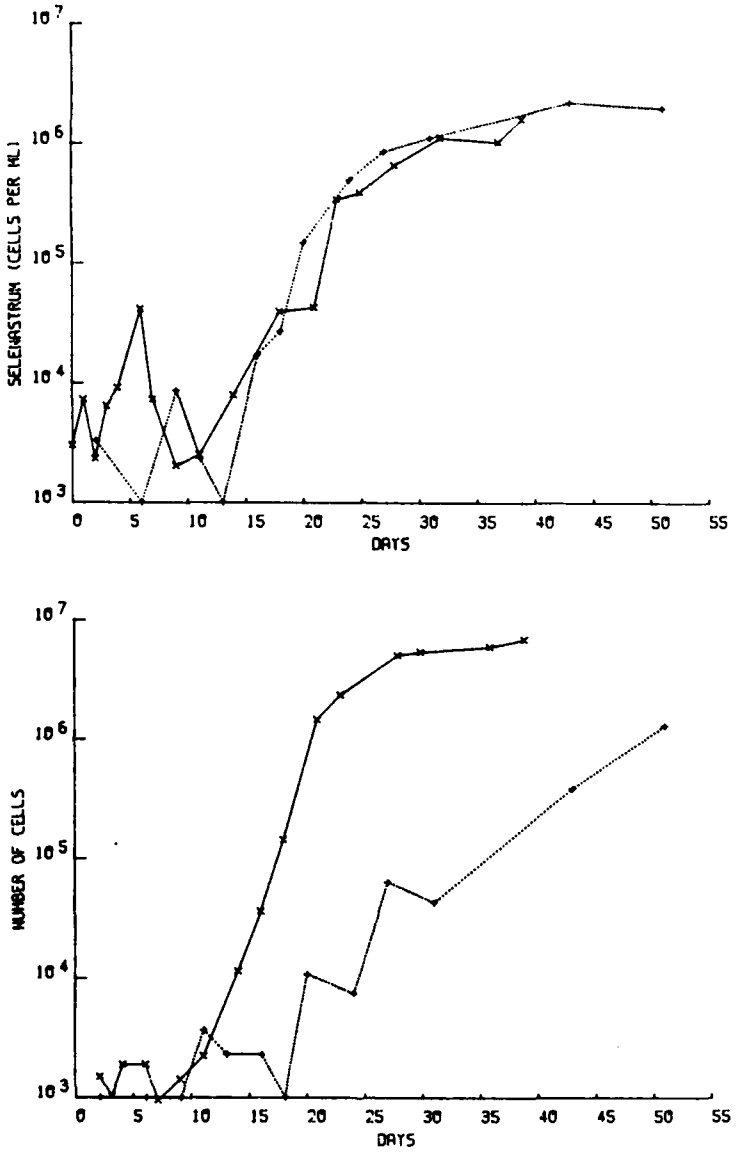

Figure 10. The growth of Selenastrum (upper graph) and Microcystis (lower graph) alone (+) and together with the other species (x) in a toluene concentration of 100% of saturation.

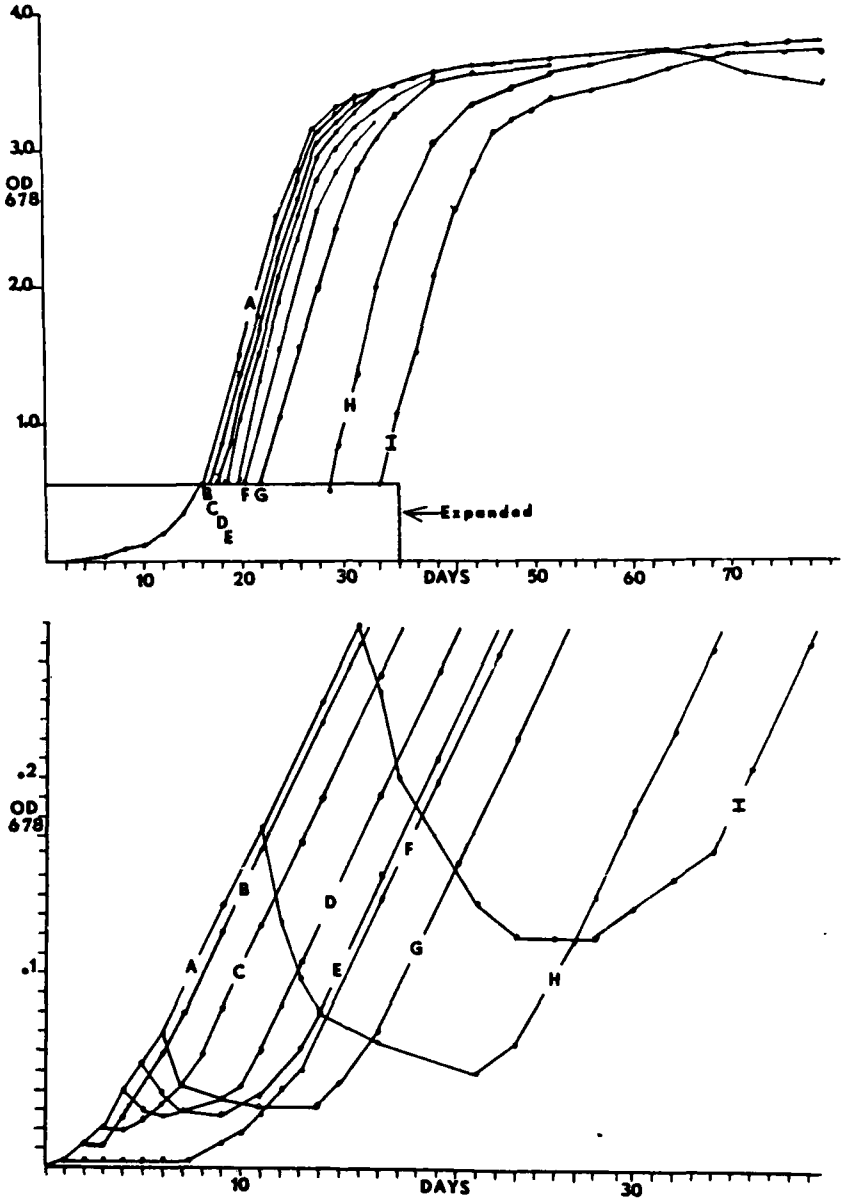

Figure 11. The influence of the stage of growth of Selenastrum on the effect of saturating concentrations of toluene in BBM medium.

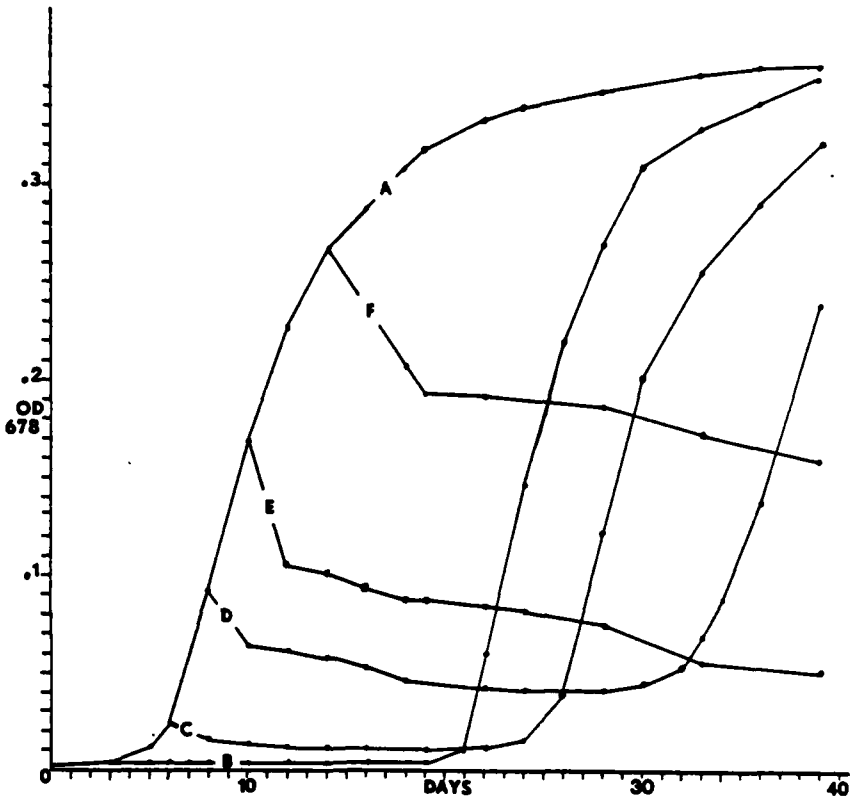

Figure 12. The influence of the stage of growth of Selenastrum on the effect of saturating concentrations of toluene in ASM medium.

were 0%, 10%, 25%, 50%, and 75% of a saturated solution. Eight replicate flasks of each concentration were prepared. Each flask was inoculated with 2.4×10^6 cells of Selenastrum. The flasks were stoppered with corks wrapped in Parafilm to prevent evaporation of the toluene. Four flasks of each concentration were inoculated with P-limited cells and four were inoculated with N-limited cells. Cell counts of each flask were made using a hemacytometer at 24, 48, and 72 hours following inoculation. At least 2 samples from each flask were counted each time.

Toluene concentrations of 50% and 75% inhibited growth of both nitrogen and phosphorus deficient algal cultures (Table 2). The cells changed color slightly, but their structure remained intact. Therefore, it could not be ascertained whether high toluene concentrations killed the cells or only inhibited the cells' growth.

At low toluene concentrations (10%) in the nitrogen limited (1:1) AAM medium, growth rate and final cell density was enhanced by the presence of toluene, while algae grown in the phosphorus deficient (23:1) AAM media were slightly inhibited by 10% toluene concentrations (Table 2). Growth of algae from both stock cultures was similar under toluene-free conditions.

Standard deviations were greater among cultures grown in the presence of low toluene concentrations. This probably reflects in part a low sample size ($n = 4$ in most cases). However, it may also result from differences in growth rates in the flasks, which would be expected if cells are growing exponentially. This is supported by the absence of such high variation among replicate flasks kept at high toluene concentrations which inhibited growth.

Table 2. The growth of nitrogen deficient (N/P = 1) and phosphorus deficient (N/P = 23) cultures of Selenastrum capricornutum in flasks containing various toluene concentrations. N = 4 in all cases (except 0% toluene, 23:1, where n=3).

Toluene Conc.	N:P Ratio	24 HRS	48 HRS	72 HRS
0%	1:1	11.244 ± 1.913	21.833 ± 3.688	26.969 ± 3.255
	23:1	9.852 ± 0.140	18.992 ± 4.427	26.577 ± 7.122
10%	1:1	10.417 ± 1.174	25.188 ± 2.301	36.313 ± 3.773
	23:1	9.695 ± 1.417	15.688 ± 2.633	18.403 ± 3.138
25%	1:1	8.403 ± 1.114	17.488 ± 6.125	23.094 ± 10.116
	23:1	9.347 ± 0.610	13.281 ± 2.987	16.956 ± 2.115
50%	1:1	8.414 ± 0.967	10.107 ± 0.567	9.063 ± 6.469
	23:1	8.500 ± 0.223	10.287 ± 1.264	8.414 ± 1.313
75%	1:1	8.639 ± 0.873	8.611 ± 0.795	9.688 ± 0.102
	23:1	7.889 ± 0.971	8.531 ± 0.616	7.222 ± 0.668

Mean cell counts + S.D.

DISCUSSION

These experiments, although admittedly preliminary, suggest that the degree of nutrient limitation and perhaps the nature of the limiting nutrient itself may alter the effect of toluene on algal growth response. In addition, toluene may affect the outcome of interactions between species.

Some of the experiments suggested that some species of algae were taking up phosphorus more rapidly from the medium than others, and, once the nutrients were depeleted, recovery after toluene addition was delayed or stopped. Besides suggesting that the results of a bioassay can be changed by delaying the time of the algal inoculation relative to toluene addition, these results have implications on the effect of toluene in natural situations. If toluene were added to a nutrient-limited lake, the dying algae may sink out of the upper waters, removing not only the algae, but also the nutrients. Recovery of this algal community would then be not so much a function of the duration of high concentrations of toluene in the water as much as the rate at which nutrients are resupplied to the system. Recovery could be in terms of weeks or months rather than the span of days suggested by the algal bioassays.

The experiments also suggested that there is a differential susceptibility to toluene among species of algae. Aside from the immediate effect of a change in algal species composition with the addition of toluene, the results suggest that the species least susceptible to toluene enjoy, at least temporarily, a competitive advantage in obtaining the available nutrients. Thus, these species may obtain a numerical advantage over more susceptible species. The results of the batch cultures used in these experiments suggest that in low nutrient income environments (as is the summer epilimnion of lakes) there will be a longer-lasting efffect on community composition that is suggested by the single-species bioassay.

In several of the experiments an increase in the number of cells over the controls was noticed. It is difficult to explain this stimulation in laboratory situations. Increases in metabolic processes such as photosynthesis or respiration have been found before, but it is difficult to conceive a method by which toluene can cause an increase in the amount of biomass produced for a given amount of nutrient. Two possibilities exist. Perhaps the toluene is contaminated with nitrogen or phosphorus, thus allowing greater accumulations than is found in the control, but contamination would not explain why 10% toluene solutions would produce a greater stimulation than 20% solutions. The second possibility is that toluene modifies the growth form of the cells, producing smaller, but more numerous cells.

There also appeared to be an interaction between nutrient limitation of the algae and toluene toxicity. Phosphorus limitation appeared to cause inhibition of growth while stimulation of growth rate was seen in nitrogen-limited cultures, suggesting that the effect of toluene may be dependent on the specific nutrient which is limiting. This observaton may become even more important when one considers that the increase of final biomass only occurred when Selenastrum was grown alone in nitrogen limiting conditions and when it was grown in the presence of Microcyctis. It may be that Microcystis modified the ambient nutrient environment in such a way that nitrogen was limiting to Selenastrum.

There was no direct test of the effect of the degree of nutrient limitation on toluene toxicity. The early tests suggest that nutrient-limited cells might be more susceptible that nutrient-saturated cells, but the results may also be interpreted by nutrient sequestering or the masking of the actual mortality by the counting techniques employed. The next experiments must be standard algal bioassays in which the cells used come from cultures of various degrees of nitrogen and phosphorus limitation. The experiments to date have suffered from the inability to directly measure mortality of the cells after toluene addiion. The next experiments will probabily use ATP and photosynthesis measurements to evaluate the immediate effect of toluene addition, raher than to rely on recovery rates, which are a functon of the rate of toluene loss from the culture, the degree of mortality, and the amount of inhibition of the surviving cells.

Certainly the results are preliminary and must be both replicated and expanded upon, but they do suggest that nutrient conditions cannot be ignored in the evaluation of bioassay results. Perhaps even more important is the possibility that the results of the bioassays may not be giving an accurate impression of the toxicity of toluene in natural, nutrient-limited environments. Experiments such as presented here can only suggest possibilities that may occur in nature, but whether they actually do occur must wait for testing in larger controlled environments than flasks.

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